The Phagocytosis of Crystalline Silica Particles by Macrophages

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Silicosis is a chronic lung disease induced by the inhalation of crystalline silica. Exposure of cultured macrophages to crystalline silica leads to cell death; however, the mechanism of cell-particle interaction, the fate of particles, and the cause of death are unknown. Time-lapse imaging shows that mouse macrophages avidly bind particles that settle onto the cell surface and that cells also extend protrusions to capture distant particles. Using confocal optical sectioning, silica particles were shown to be present within the cytoplasmic volume of live cells. In addition, electron microscopy and elemental analysis showed silica in internal cellular sections. To further examine the phagocytosis process, the kinetics of particle uptake was quantified using an assay in which cells were exposed to ovalbumin (OVA)-coated particles, and an anti-OVA antibody was used to distinguish surface-bound from internalized particles. Fc receptor-mediated uptake of antibody-coated silica particles was nearly complete within 5 minutes. In contrast, no OVA-coated particles were internalized at this time. After 30 minutes, 30% of bound silica was internalized and uptake continued slowly thereafter. OVA-coated latex beads, regardless of surface charge, were internalized at a similarly slow rate. These results demonstrate that macrophages internalize silica and that nonopsonized phagocytosis occurs by a temporally, and possibly mechanistically, distinct pathway from Fc receptor-mediated phagocytosis. Eighty percent of macrophages die within 12 hours of silica exposure. Neither OVA coating nor tetramethylrhodamine isothiocyanate labeling has any effect on cell death. Interestingly, antibody coating dramatically reduces silica toxicity. We hypothesize that the route of particle entry and subsequent phagosome trafficking affects the toxicity of internalized particles.

Keywords: silica; phagocytosis; cell death

Silica is the most abundant element on Earth, and crystalline silica dioxide is the predominant form of silica in the environment. As a result, crystalline silica is also the major component of airborne dust generated by wind, manufacturing, or demolition. Chronic inhalation of crystalline silica leads to silicosis, a disease characterized by inflammation and fibrosis of the lung (1). When environmental particles are inhaled, the particulate matter is deposited in the lungs. Silica appears to affect epithelial cells, fibroblasts, and lung macrophages (2–5). Prolonged deposition of particles in the alveoli or bronchioles leads to lung inflammation, the formation of fibrotic scar tissue, and degradation of the mucociliary escalator (1). Even though

CLINICAL RELEVANCE

The toxicity of silica particles is presumed to be related to their uptake into cells, but no data show this. We prove that most particles are internalized but that the process differs from Fc-mediated phagocytosis.

silicosis is preventable through the use of respirators, this simple expedient is often not used, either through neglect or circumstances, as in the collapse of the World Trade Center (6). Of the 100,000 workers in the United States exposed to crystalline silica, about 250 die each year from silicosis (USDHHS statistics).

Exposure of cells to crystalline silica is known to cause cell death, and it is presumed that this toxicity is the underlying cause of silicosis (1, 4, 7). Although the interaction of silica with cells has been intensively studied, the molecular mechanisms underlying this toxicity are not well understood (8). There is evidence that silica causes the production of free radicals and reactive oxygen species (9–11) and that some cells die due to activation of the apoptotic cascade (12–14). However, the actual mechanism of silica-induced cell death is still unclear.

There are three major pathways by which biological particles are recognized and internalized by immune cells. Antibody-coated particles are recognized by the Fc receptor, complement-coated particles by the complement-receptor, and particles such as yeast by the mannose receptor (15, 16). The molecular mechanisms by which cells interact with nonbiological particles such as silica, however, are not clear. One hypothesis is that the scavenger receptor (SR-A) is responsible for binding silica and other uncoated particles (17–20). The three major members of the scavenger receptor family are SR-AI and SR-AII, derived from alternative splicing, and MARCO (macrophage receptor with collagenous structure). These receptors all share collagen-like and cysteine-rich domains in their extracellular regions. At the collagen-like domain, a lysine cluster forms a positive-charged groove to interact with negative-charged particles, such as LDL (21, 22). Silica has a negative surface charge, and this has led to the hypothesis that silica is recognized by a similar mechanism (23, 24). When cells are pretreated with scavenger receptor blockers (24–26), or when alveolar macrophages from an SR-A knockout mouse are exposed to particles (17, 27), binding and toxicity are reduced but not eliminated. These results suggest that there may be multiple mechanisms of binding and uptake of particles.

When biological particles bind to the surface of cells, sometimes they are internalized via the process of phagocytosis. Previous work has shown that environmental particles bind to cells, but whether they are phagocytosed is less clear. Electron microscopy of sections of lung tissue and cultured cells shows internal silica particles, but this technique does not allow quantification of uptake or the relationship of uptake to cell death (28, 29). Flow cytometry has been used extensively to characterize the association of opsonized (antibody-coated) and non-opsonized (uncoated or nonspecific protein-coated) particles with cells, but the assays used do not distinguish between

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surface-bound and internalized particles. Furthermore, much of the work on nonopsonized particles focuses on latex beads and titanium dioxide particles, and less on silica (17, 18, 30–32). Thus, the cellular fate of the silica particles has not been directly determined. This issue is critical to understanding silica toxicity because if particles were mostly on the surface of cells, then toxicity would likely be triggered at the plasma membrane. Internalization would suggest numerous other potential cytoplasmic targets. We have developed a quantitative phagocytosis assay that allows us to compare the internalization of silica and other nonbiological particles with uptake by the Fc receptor-mediated pathway. The results show that nonopsonized particles are internalized slower than opsonized particles, but silica toxicity is dependent upon the route of entry of the particles.

MATERIALS AND METHODS

Chemicals

All chemicals were from Sigma (Sigma Chemical Co., St. Louis, MO) unless otherwise noted.

Cell Lines

The mouse alveolar macrophage cell line MH-S (ATCC CRL-2019) and mouse peritoneal macrophage cell line RAW 264.7 (ATCC TIC-71) were cultured at 37°C with 5% CO₂ in RPMI-1640 media (Media Tech, Pittsburgh, PA) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 55 µM 2-mercaptoethanol, 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 µg/ml ampicillin, and 100 µg/ml dihydrostreptomycin sulfate.

Particle Types

1. Crystalline silica: Alpha-quartz particles (Min-U-Sil 5; US Silica, Berkeley Springs, WV)
2. Spherical silica (Allsphere silica; Alltech Associates, Deerfield, IL). Note that most experiments were performed with crystalline silica, but similar results have been found using spherical silica.
3. Plain, Amino, and Carboxy Latex Beads (Polysciences, Inc., Warrington, PA)

Particle Coating Methods

Coating silica and plain latex beads with ovalbumin. Two milligrams of crystalline silica or 100 µl plain 1- or 3-µm latex beads were coated by incubating them in 1 ml of 10 mg/ml ovalbumin (OVA) in Particle Coating Buffer (PCB) (1.8 mM Na₂CO₃, 3.2 mM NaHCO₃, 135 mM NaCl), pH 9.5, for 90 minutes at 37°C while tumbling.

Coating charged latex beads with OVA. Positive- or negative-charged latex beads were coated with OVA according to manufacturer's instructions (Polysciences 238F and 238G protocols) using carbodiimide for carboxy latex beads and glutaraldehyde for amino latex beads.

Coating silica and latex beads with antibody. Rabbit anti-chicken egg albumin (Immunology Consultants Laboratory, Newberg, OR) at 0.2 mg/ml in PBS was added to OVA-coated particles and tumbled for 90 minutes at 37°C.

For all three protocols, particles were centrifuged and washed three times in PBS before use.

Labeling of silica with tetramethylrhodamine isothiocyanate. Two milligrams of crystalline silica was added to 1 ml of PCB containing 50 µg/ml of tetramethylrhodamine isothiocyanate (TRITC)-R (Sigma) and tumbled for 8 hours at 37°C. The particles were then centrifuged and washed with 50 mM NH₄Cl, pH 7.25, for 2 hours at 37°C while tumbling to stop the reaction. Particles were then washed six times in PBS and stored in PBS at 4°C.

Live Cell Particle Binding Assay

Cells were plated at 1×10^5 cells/cm² in a Delta T glass-bottom culture dish (Bioptechs, Inc., Butler, PA) in RPMI-1640 complete media and allowed to adhere overnight, and then media was replaced with

bicarbonate-free RPMI media supplemented with 25 mM HEPES, pH 7.5 (BF-media). Cells were maintained at 37°C in an ambient atmosphere incubator for 15 minutes and then moved to the microscope stage, where they were maintained at 37°C by a Delta T controller (Bioptechs). Imaging was initiated and then exposed to 15 µg/cm² silica. Differential Interference Contrast (DIC) images were acquired at 30-second intervals using automation routines controlled by OpenLab software (Improvision, Inc., Portage, MI). Images were processed using ImageJ (33). Images were also converted to Quicktime movies (Apple, Inc., Cupertino, CA) to observe time-lapse particle binding.

Confocal Particle Imaging Assay

Before exposing cells to TRITC-labeled or unlabeled silica, cells were plated in Delta T glass bottom culture dishes (Bioptechs) in BF-media containing 50 µM 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Inc., Eugene, OR) for 30 minutes at 37°C in an ambient atmosphere incubator. Media was then replaced with fresh BF-media and the cells were imaged using a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). Simultaneous DIC, fluorescein, and rhodamine fluorescence, or DIC, fluorescein, and reflectance image stacks were acquired at time 0 hours and after 1 hour of incubation. Approximately 40 optical sections totaling 9 µm were taken from the top to the bottom of each cell. X-Y-Z stack resectioning to determine particle localization within the cell volume was performed using the Leica software.

Electron Microscopy

A quantity of 1.5×10^5 MH-S macrophage cells was plated on aclar in Bioptech dishes overnight. RPMI 1640 complete media was replaced with CO₂-independent media (Invitrogen, Inc., Carlsbad, CA) and cells were either treated with 50 µg/cm² of silica for 4.5 hours or left untreated. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3 for 30 minutes at room temperature. Fixed cells were washed and treated with 1% Osmium tetroxide in 0.1 M sodium cacodylate for 30 minutes at 4°C. After a wash, cells were dehydrated in a graded ethanol series with propylene oxide as a clearing agent. *En bloc* staining with 1% Uranyl Acetate was performed in 70% ethanol for 1 hour at 4°C. Cells were embedded in an epoxy resin mixture consisting of SPI-PON 812, Araldite 506 and DDSA. Ultrathin sections of approximately 10 nm were cut using a diamond knife and stained with 4% Uranyl acetate in 50% ethanol and 2.5% lead citrate. Elemental composition of particles was determined by electron probe X-ray microanalysis performed in scanning transmission mode at 100 keV on a Zeiss EM910 electron microscope (Zeiss, Göttingen, Germany), equipped with an Oxford ExL II analytical system and 30 mm² Si(Li) detector (Oxford Instruments, High Wycombe, Bucks., UK).

Quantitative Phagocytosis Assay

Before particle exposure, cells were plated at 1×10^5 cells/cm² on 25-mm round glass coverslips in 30-mm dishes in RPMI 1640 complete media and allowed to adhere overnight. Media was replaced with BF-media for 15 minutes at 37°C in an atmospheric incubator and then chilled on ice for 10 minutes. OVA-coated, antibody-coated, or uncoated particles were added to cells (15 µg/cm² of silica, or 40 µl of 2.5% latex beads) and then the plate was centrifuged at $300 \times g$ for 5 minutes. The time zero coverslip was immediately fixed with 4% formaldehyde for 6 minutes at 25°C and the remaining plates were placed in the 37°C atmospheric incubator and fixed at various time intervals. After fixation, the media was replaced with 50 mM NH₄Cl, pH 7.25, for 3 minutes, and then the media was changed to PBS for 3 minutes. PBS was replaced with 200 µl of 1:800 rabbit anti-OVA in PBS (Immunology Consultants Laboratory, Newberg, OR) and incubated for 35 minutes. Primary antibody was then removed and replaced with 200 µl of 1:150 FITC-conjugated goat anti-rabbit antibody in PBS (Jackson ImmunoResearch, West Grove, PA) for 35 minutes. Secondary antibody was then removed and the coverslips were washed twice with PBS and once with water before mounting in 10% DABCO/2.5% MOWIOL mounting media. Particles that were pretreated with anti-OVA antibody (Ab-silica and Ab-beads) were stained with secondary antibody only. Imaging was performed with a $\times 63$ oil immersion objective on a Zeiss Axiovert 200M microscope using a Hamamatsu ORCA-ER camera (Hamamatsu, Bridgewater, NJ).

The antibodies are not membrane permeable in fixed cells, so only external particles become fluorescent. To quantify particle internalization, labeling of particles by antibody was determined visually. The number of surface-bound particles was counted from the fluorescence images, and the total number of particles from the DIC images. The amount of phagocytosis per cell was quantified by subtracting the number of fluorescent particles from the total number of particles to derive the number of internalized particles. At each time point, this number was divided by the total number of particles to derive the percent phagocytosis. The error bars depict standard error from three separate experiments.

Cell Death Assay

Cells were plated at 1×10^5 cells/cm² in a Delta T glass-bottom culture dish (Bioptechs) in RPMI 1640 complete media and allowed to adhere overnight, after which media was replaced with BF-media for 15 minutes at 37°C in an ambient atmosphere incubator. The cells were then moved to the microscope stage and exposed to 15 µg/cm² silica and 0.1 µg/ml Propidium Iodide (PI). The cells were maintained at 37°C on the microscope stage by the Delta T stage incubator (Bioptechs), and DIC and fluorescence images were acquired at 15-min intervals using automation routines controlled by OpenLab software (Improvision, Inc., Portage, MI). Images were processed using ImageJ (33), and 300 cells from each time point were scored manually for nuclear fluorescence. Since apoptotic cells eventually undergo secondary necrosis, the percentage of PI-positive cells is a measure of both necrotic and apoptotic cell death. Images were also converted to Quicktime movies (Apple) to observe time-lapse particle binding and the induction of cell death.

RESULTS

Silica-Induced Cell Death Is Time and Concentration Dependent

It is well known that silica induces cell death in macrophages, but the cells are usually exposed to concentrations of 50 µg/cm² or higher. To study the trafficking of individual particles, macrophages must be exposed to lower concentrations of silica to be able to clearly distinguish the number of particles associated with each cell. MH-S and RAW264.7 macrophages were exposed to varying concentrations of silica to determine the lowest concentration at which particles are toxic to cells. At 0, 4, and 10 hours, images were taken to quantify the proportion of dead cells. Concentrations of silica as low as 15 µg/cm² caused greater than 60% of cells to die by 10 hours (Figure 1 and data not shown). By examining cells treated with low amounts of silica, it appears that cells with more than three to

five bound particles usually die, while cells with less than three particles remain alive. Based on these results, a concentration of 15 µg/cm² of silica was used for all subsequent experiments. This represents an average of eight particles per cell. Latex bead concentrations were adjusted to present cells with similar numbers of particles.

Macrophages Gather Particles by Extending Protrusions

To learn how cells interact with silica particles, time-lapse video microscopy of macrophages before and after silica or latex bead exposure was performed. When silica particles are added to the media, they rapidly settle to the bottom of the dish. Some particles land directly on the apical cell surface and remain adhered, while others land on the surface near the cells. After a few hours, there is an increase in the number of particles on the cells and a clear zone is created around each cell as these distant particles are captured (Figure 2A). The clear zones are formed as cells randomly extend membrane protrusions up to about 7 µm away. When these protrusions encounter a particle, the membrane moves underneath the particle and then the particle is either transported along the surface of the extension, or the membrane is retracted, bringing the particle to the cell body (Figures 2B and 2C; *see also* the movie in the online supplement). Thus, cells actively recruit nearby particles by movement and membrane extension. Once associated with the cell, particles do not appear to be subsequently lost.

Silica Particles Are Phagocytosed by Macrophages

Opsonized (antibody-coated) and nonopsonized (uncoated or nonspecific protein-coated) nonbiological particles such as titanium, diesel exhaust, and silica have been extensively studied by flow cytometry to determine their association with macrophages (15, 20, 28–30, 34–36). Although it is clear that macrophages bind quartz silica (17, 24–27), there is little information about the extent and timing of particle phagocytosis. To directly address this question, confocal microscopy was used to visualize the three-dimensional localization of particles associated with cells. The cytoplasm of MH-S macrophages was labeled with 5-chloromethylfluorescein diacetate (CMFDA), and then the cells were exposed to silica that was fluorescently labeled with tetramethylrhodamine. After 1 hour of incubation, confocal optical sectioning of the cells was performed to generate a volumetric data set. Red fluorescent particles were seen surrounded by the

Figure 1. Silica-induced cell death is time and concentration dependent. MH-S macrophages were exposed to increasing concentrations of silica particles in the presence of propidium iodide (PI). The cells were imaged at various times to determine the toxicity of the different particle loads by measuring nuclear PI staining. The minimal concentration of silica that induced significant cell death was 15 µg/cm². This concentration of silica was used in all subsequent experiments. Latex beads did not induce cell death at any concentration (data not shown).

Figure 2. Macrophages gather particles by extending protrusions. MH-S or RAW264.7 macrophages were exposed to silica particles or 3- μ m plain latex beads and imaged using differential interference contrast (DIC). Images were captured every 30 seconds and then assembled into a time-lapse movie. (A) Silica particles rapidly land on and near macrophage cells. Over time, a majority of nearby particles were located on the cells, creating clear zones around each cell (78' panel). Pseudopods on macrophages extend out, up to about 7 μ m away, to capture (B) 3- μ m latex beads and (C) silica particles and move them to the cell surface. Either an arrow or an arrowhead is used to indicate the route of specific particles. Scale bars are 10 μ m for A, and 5 μ m for B and C.

green fluorescent cytoplasm of macrophages when re-sectioning of an X-Y-Z-stack was performed (Figure 3A, and movie in the online supplement). Red particles bound to the surface were not surrounded by green cytoplasm. To verify that the fluorescent labeling of the particles was not affecting the uptake process, unlabeled particles were examined using confocal reflection mode. Unlabeled silica particles were also seen surrounded by green cytoplasm, verifying that they are phagocytosed as well (Figure 3B, and movie in the online supplement).

Elemental X-Ray Analysis Confirms the Presence of Silica Particles Inside Macrophages

An alternative way to demonstrate the internalization of silica is by electron microscopy (28, 29). MH-S macrophage cells were exposed to silica particles for 2 hours before the cells were fixed, sectioned, and prepared for transmission electron microscopy. Holes were frequently found in the sections of silica-treated cells that are presumed to be due to particles being knocked out of the section by the diamond knife during sectioning. Periodically, a particle remained associated with the section through processing and could be seen in the EM image. Ultrathin sections showed electron dense particles within the cells (Figure 4A). To confirm that this particulate matter was silica, it was subjected to X-ray microanalysis. Both particles 1 and 2 show

a characteristic peak at 1.741 keV, indicating the presence of silicon (Figure 4B, and data not shown). Some large silica particles were displaced during the course of sectioning, leading to the appearance of knife marks (Particle 2, Figure 4A). Elemental analysis on a control area (Figure 4A) showed much lower silicon peaks as compared with the particles (Figure 4B), confirming that these peaks do not arise from embedding medium or stain. These data further support the presence of silica within the cells.

Kinetics of OVA-Coated Particle Phagocytosis by Macrophages

There has been little investigation into the mechanism by which uptake of nonopsonized particles occurs. Although the confocal and electron microscopy assays confirm that nonopsonized silica particles are taken up by cells, it would be difficult to use these assays to quantify the kinetics of particle internalization. Therefore, an assay was developed that uses the same principle as commonly used for measuring Fc receptor-mediated phagocytosis (37–39). Silica particles were coated with a nonspecific glycoprotein (OVA) and added to cells. Cells were fixed but not permeabilized at various time points and fluorescently stained with an OVA-specific antibody. Since antibodies cannot penetrate intact cells, the antibody only binds to the external particles,

Figure 3. Silica particles are phagocytosed by macrophages. (A) Tetramethylrhodamine isothiocyanate (TRITC)-labeled silica or (B) unlabeled silica was added to 5-chloromethylfluorescein diacetate (CMFDA)-stained macrophages, and the cells were incubated for 1 hour. The live cells were then imaged by confocal microscopy in (A) fluorescence mode or (B) reflectance mode. The CMFDA stains the entire cytoplasm green so that the silica particles can be localized within the three-dimensional cell volume. X-Y-Z re-sectioning of the data shows that (A) red or (B) white particles are surrounded by green cytoplasm in the side and bottom views of each re-sectioned stack. Scale bars are 2 μ m in A and 1 μ m in B.

Figure 4. Elemental X-ray analysis confirms the presence of silica particles inside macrophages. (A) Scanning transmission electron micrograph of a MH-S macrophage exposed to silica showing the presence of silica particles of varying sizes. Both particles 1 and 2 as well as the control area were subjected to X-ray microanalysis. (B) X-ray spectrum from particle 1 to confirm the presence of silicon (Si). A peak was seen at 1.741 keV, corresponding to the Si K α peak. Similar results were seen for particle 2 (not shown). (C) X-ray spectrum from cytoplasmic region of cell to determine background levels of Si from fixative and embedding material. The peak seen at 1.741 keV was much smaller than that of particle 2.

allowing internalized and external particles to be distinguished (Figures 5A and 5B). The total number of particles can be determined directly by DIC imaging.

Using this assay, the uptake rate of nonopsonized (OVA-coated particles) and opsonized (the same particles treated with anti-OVA antibody) particles was determined. Consistent with previous work, within 5 minutes almost all antibody-coated latex beads were internalized (38, 40, 41). The same result was found for antibody-coated silica, indicating that the uptake of

Figure 5. Kinetics of phagocytosis of particles by macrophages. Ovalbumin (OVA)-coated 3- μ m carboxy latex beads (A) or silica particles (B) were added to macrophages, and then the cells were fixed at intervals and stained with antibody against OVA. Internalized particles (arrows) are unstained, while external particles are fluorescent (arrowheads). (C) Quantification of the kinetics of uptake of opsonized and nonopsonized particles. MH-S macrophage cells rapidly internalize antibody-coated 3- μ m carboxy latex beads and silica. Nonopsonized, OVA-coated silica, plain (uncharged) latex beads, amino latex beads, and carboxy latex beads are phagocytosed significantly slower than opsonized particles. Scale bars are 5 μ m. Open circles, Ov-Silica; solid circles, Ab-Silica; solid squares, 1- μ m Ov-Carboxy Latex Beads; closed triangles, 3- μ m Ov-Carboxy Latex beads; open squares, 1- μ m Ov-Plain Latex beads; solid diamonds, 1- μ m Ov-Amino Latex beads; open triangles, 3- μ m Ab-Carboxy Latex beads.

silica by the Fc receptor is similar to the uptake of other types of opsonized particles. In contrast, there was no internalization of nonopsonized particles at this time. Within 15 minutes, nonopsonized particles had begun to be internalized and, by 30 minutes, there was about 30% uptake of all nonopsonized particle types. Uptake plateaued at about 50% of particles internalized by 180 minutes. Interestingly, OVA-coated carboxy (negative-charged), amino (positively charged) latex beads, and OVA-coated silica were all internalized on a similarly slow time scale (Figure 5C). This result indicates that particle surface charge does not affect the recognition and internalization process (Figure 5C). In addition, the uptake of 1- μ m and 3- μ m

latex beads occurred on a similar time scale (Figure 5C), indicating that the heterogeneous size of the crystalline silica is not a factor in the uptake rate.

One potential concern with this internalization assay is that coating particles with OVA might affect the uptake of silica or latex particles. Several control experiments indicate that this is not the case. First, the uptake of unlabeled and TRITC-labeled silica was measured using the confocal microscopy assay. The proportion of both types of nonopsonized silica internalized after 1 hour was consistent with the results found using the OVA antibody assay (data not shown). Second, if soluble ovalbumin was added to the BF media before the addition of uncoated or OVA-coated silica particles, there was no decrease in the number of particles bound per cell nor the amount of particles internalized over time.

Coating Silica Particles with Antibody Dramatically Reduces Their Toxicity

Previous data demonstrated that exposure of cultured macrophages to silica leads to the appearance of a number of markers for cell death (13, 14, 24–26). Propidium iodide staining was used as a marker for cell death to determine if other particle types used in uptake assays were toxic. Exposure of MH-S macrophage cells to media alone (Figure 6A), or to 3- μ m plain (uncharged), amino (positive-charged), or carboxy (negative-charged) latex beads, resulted in little or no cell death (Figure 6C). Within 12 hours of silica exposure, about 80% of macrophages died (Figure 6B). Coating silica particles with OVA or labeling with TRITC did not significantly alter the induction of cell death. When silica and latex beads were coated with bovine serum albumin (a nonglycosylated protein), casein (a phosphoprotein), or histone IIA (a positive-charged protein) instead of OVA, silica-induced cell death was similar to uncoated and OVA-coated silica data (data not shown). Surprisingly, antibody-coated silica was dramatically less toxic to cells (Figure 6D). Therefore, the receptor used for particle binding, or some aspect of the downstream vesicle trafficking pathway, has a dramatic effect on the toxicity of these particles.

Fc Receptor Cross-Linking Does Not Suppress Silica-Induced Cell Death

Since antibody-coated silica suppressed cell death, it is possible that the activation of macrophages by cross-linking of the Fc receptors is sufficient to prevent cell death induced by silica. To test this hypothesis, cells were pretreated with antibody-coated latex beads and then subsequently challenged with silica particles. Different concentrations of antibody-coated 1- μ m latex beads or antibody-coated 3- μ m spherical silica (not shown) were added to cells to promote cross-linking of the Fc receptors. After 15 minutes, uncoated 3- μ m silica was added to these cells and cell death was measured. In all cases, pretreatment of cells with opsonized particles did not prevent silica-induced cell death (Figure 7). Thus, the suppression of toxicity by antibody-coating the silica particles does not appear to be due solely to activation of the macrophages by Fc receptor cross-linking.

DISCUSSION

The fact that cells interact with environmental particles like silica has been known for some time, and it has been presumed that these particles are internalized. However, the only data that directly demonstrate internalization comes from TEM images of particles in sections of cells exposed to silica (28, 29). This methodology, however, is not amenable to quantitative analysis

Figure 6. Coating silica with antibody dramatically reduces their toxicity. Macrophages were exposed to particles and PI was used to quantify the extent of cell death. A minimal number of cells not exposed to particles (A) or exposed to 3- μ m carboxy-latex beads (C) become membrane permeable by 12 hours. However, silica (B) induces membrane permeability in about 80% of cells by 12 hours. (D) Quantification of death in cells exposed to a variety of particle types. When macrophages were exposed to uncoated, OVA-coated, or TRITC-labeled (nonopsonized) silica, cell death is induced to a similar extent. When macrophages are exposed to antibody-coated (opsonized) silica, cell death is significantly suppressed. Scale bars are 5 μ m.

of the internalization of particles. Most work has used flow cytometric measurement of right angle scatter to detect cell-associated particles (17, 18, 30–32). However, the particles scatter light whether they are inside or outside the cell, so the

Figure 7. Fc receptor cross-linking does not prevent silica-induced cell death. Macrophages were exposed to increasing concentrations of antibody-coated 1- μ m latex beads to promote Fc receptor cross-linking and then, after 15 minutes, 3- μ m uncoated spherical silica was added to the same cells. PI was used to quantify cell death. The pretreatment with opsonized latex beads did not prevent silica-induced cell death.

increase in side scatter essentially measures the total association of particles with cells. One way to estimate the proportion of internalized particles is to treat cells with cytochalasin D, an inhibitor of actin filament elongation that primarily inhibits phagocytosis but not binding of particles (31). Consistent with the idea that some particles are internalized, treatment of cells with cytochalasin D decreased, but did not eliminate, the right angle scatter increase of cells treated with silica (18, 29). To examine to what extent particles are internalized, we have developed assays that allow us to directly observe and quantify the internalization process.

Cultured macrophages extend protrusions and move randomly on glass or plastic surfaces. Since particles do not release any chemical signals, it is presumed that, unlike bacteria, macrophages *in vivo* or *in vitro* encounter particles accidentally. Whether the particles settle onto the surface or make contact through membrane protrusions, the particles appear to stick avidly to the cell surface. When a pseudopod or lamellipod encounters a particle, the protrusion extends underneath and beyond the particle, and then draws the particle back to the cell body (Figure 2). Particles do not seem to fall off of the cells once they are bound, and we have not seen any evidence of particle exocytosis. We have used several techniques to verify and quantify the internalization of nonopsonized silica and latex particles. Using light microscopy, it is straightforward to visualize the amount and diverse size of crystalline silica bound to each cell. However, to determine if these particles are phagocytosed, several assays were used. Examining the cell volume by confocal optical sectioning or looking at EM thin sections both showed particles inside cells. However, these assays would be cumbersome for routine quantification. Therefore, a third assay was developed using OVA-coated latex beads and silica. These silica particles caused the same cellular toxicity as uncoated silica particles, so the coating process does not seem to markedly alter the silica. Adding soluble OVA to the media before exposure of nonopsonized particles also does not affect binding or internalization, indicating that the OVA does not play a role in the binding or uptake process. Consistent with the other uptake assays, within 60 minutes, 40% of particles were internalized. With this information, previous flow cytometry data can be seen as a measure of total particle association, comprising both internalized and surface bound particles, but the data

overestimates the amount of particle internalization. However, it is not yet known if internalization is necessary for toxicity.

Two surprising observations have come from direct measurement of the internalization of particles. First, internalization of either nonopsonized silica or latex is much slower than Fc receptor-mediated internalization of either particle type. The number of bound particles per cell remained fairly constant over the course of the assay, regardless of particle type. It is only the internalization of nonopsonized silica and latex particles that was slow, and the flow cytometry assay cannot distinguish between the internal particles and external particles. This may indicate that internalization of nonopsonized particles is, in some way, mechanistically different from Fc receptor-mediated uptake of opsonized particles. It should be noted that we have not measured particle-cell interaction in the presence of any shear forces, so it is possible that the force of interaction may differ depending upon the particle type.

The second key result is that the toxicity of silica is dramatically reduced if the particles are opsonized. This effect is not due to the fact that the particles are coated with protein since coating silica with OVA, bovine serum albumin, casein, or histone IIA had no effect on particle toxicity. It is interesting that coating silica with negative- or positive-charged proteins does not change their toxicity. Thus, it is more likely that the alteration in toxicity is due to targeting the particles to the Fc receptor-mediated uptake pathway. One hypothesis to explain this result would be that the opsonized particles activate the macrophages and that this activation somehow protects them from the toxic effects of silica. When Fc receptors are cross-linked by binding to opsonized particles, Syk and Src tyrosine kinases are phosphorylated and the cell becomes activated leading to an oxidative burst and expression of TNF and MIP-2 (42, 43). To test this hypothesis, cells were activated with antibody-coated latex beads and then exposed to silica. Surprisingly, this pretreatment was not protective. Both Fc receptor activation and silica have been shown to cause an increase in reactive oxygen species (ROS) production by macrophages (11, 18, 35, 36). If ROS were damaging to the cell, then it would seem likely that both particle types would be toxic, and that antibody-coated silica might be even more toxic. Since both particle types lead to ROS production, and only nonopsonized silica is toxic, either ROS does not play a role in the toxicity, or there is

something different about the type of ROS, or the timing or location of its production.

These results also raise the interesting possibility that the route of entry of particles into the cell affects their subsequent toxicity because some aspect of the signaling pathway associated with nonopsonized particle uptake, or some difference in vesicle trafficking of phagosomes after uptake, alters toxicity. How this relates to the exposure of inhaled particles to mucus, ECM proteins, and serum in the lung is not yet clear. Presumably, these molecules could coat the particles but, like OVA, this may not alter the particle's toxicity. It will be important to establish that alveolar macrophages isolated from bronchoalveolar lavage show the same particle uptake properties as the cultured cell lines that we have used.

The mechanism by which the silica particles become bound to the surface of the cell is not clear. It seems unlikely that cells possess a specific receptor for nonbiological materials like silica, titanium, and latex. The literature suggests receptors of the scavenger receptor family-A play a role in silica binding and cell death (17–20). However, it is not clear that these receptors are solely involved since binding and subsequent silica-induced cell death still occur in the absence of these receptors (17, 27). We have found that the binding and rate of internalization of plain, negative-charged, or positive-charged latex beads are all about the same. In addition, nonopsonized silica are internalized at a similarly slow rate. Recently, we have found that manufactured amorphous silica spheres behave in a similar manner to crystalline silica (data not shown). Thus, neither surface charge nor composition of particles has a dramatic effect on binding or internalization. Moreover, these experiments were performed in serum-free media. Therefore, media components are not promoting internalization. These results appear to be inconsistent with the idea of scavenger receptor interacting with a negative-charged surface of the particle, although it is possible that there are negative-charged surface patches even after protein coating.

Whether particle internalization is required for cell death is currently unknown. Internalization is prior to any of the known events associated with the cell death pathway (14). It will be important to find a way to block uptake without blocking binding to answer this question. It has been noted that, if silica is killing macrophages, and the macrophages are responsible for eating dying cells, then the lung would likely enter a futile cycle in which silica-exposed macrophages undergo cell death while more macrophages are attracted to the site. Whether internalized silica, which is presumably inside the phagosomal membrane of dying macrophages, is itself toxic to cells is unknown; it will be important to answer this question to get a better understanding of the events that occur in the lung that lead to silicosis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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